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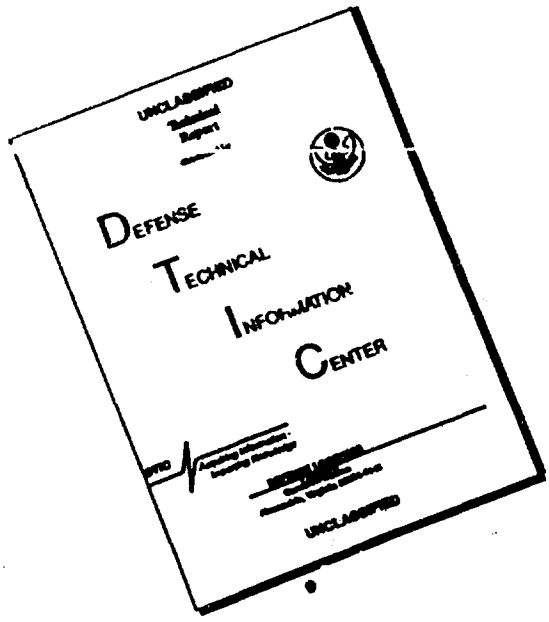

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INVESTIGATION OF BOTULIN TOXINS AND TOXOIDS WITH THE GEL FILTRATION METHOD

Report II.

Separation of Crude Type A Botulin Toxins Via Filtration Through Sefadex G-200 Gel

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One of the parameters characterizing a chemical substance is its molecular weight. However, there is no unified opinion in literature concerning the molecular weight of botulin toxins and, in particular, of botulin toxin type A.

Putnam and coauthors (1946), Kegeles (1946), Heyningen (1950), Mesrobyanu and Puenescu (1963), Vorob'yev and coauthors (1947) considered the molecular weight of botulin toxin type A as equal to 900,000-1,300,000. According to the data of Buchler and coauthors (1947), it equals 45,000. The same figure is cited in the monograph of Morgunov (1959).

Gerwing and coauthors (1965) fractionated botulin toxin type A via its precipitation with ammonium sulfate, and separating it on DEAE-cellulose. During the last purification phase the concentrated toxin was dialyzed against a guanidine acetate buffer (pH 4.5) as a diaggregation factor. Thus purified, the type A botulin toxin had a sedimentation constant of 0.93S and molecular weight of 12,200. It was thought that the difference

between the figures of molecular weight of the type A botulin toxin obtained by above authors and values cited in the literature, is caused by polymerization phenomena which have not been taken into account by previous investigators.

The considerable discrepancies in obtained values can apparently be explained from the point of view of Ambache (1948), who attempted to find out, how very large molecules of botulin toxins can be absorbed in the gastrointestinal tract. He was the first to advance a hypothesis that the botulin toxin molecules disintegrate into smaller units, which are capable of entering from the lumen of the gastrointestinal tract into the general blood circulation, and even into the cells. The Ambache hypothesis was experimentally confirmed by Wagman and Bateman (1951, 1953, 1954, 1963). By means of ultracentrifugation and diffusion, the molecular weight of the crystalline type A botulin toxin, at pH 3.8-4.4, was established by these authors as equalling 900,000-940,000. At pH 7.5 in a 0.05 M phosphate buffer, the type A botulin toxin became dissociated into fractions which were similar as to their amino acid composition and toxicity. The low-molecular fraction with a 7S sedimentation constant and molecular weight of 158,000 comprised about 14% of the total toxin quantity. A more rapid dissociation took place in an alkaline medium at pH 9.2. The authors obtained fragments with a molecular weight up to 2,900-3,800 which still possessed toxic properties.

As seen from the data of researchers working with botulin toxin type A they encountered molecules of various size. It seemed of interest to ascertain the value of the molecular weight of toxin molecules in crude preparations which are used for the preparation of antigens for active immunization; it might be of theoretical as well as practical importance for the substantiation of various methods of purification and concentration of botulin toxins and toxoids. Previous works were carried out with highly purified or crystalline botulin toxins. The purification could apparently contribute to the processes of dissociation or aggregation. It seemed to us expedient to carry out experiments with a crude type A botulin toxin.

For investigation of the crude type A botulin toxin we employed filtration through sefadex gel, based on the principle of molecular strainers. Upon passing a molecular mixture through a column filled with sefadex, there appear in the outflowing solution at first the largest molecules, and then -- the remaining molecules in the order of decreasing size (Flodin, 1962). Therefore, the method of filtration in gel is used for determination of the molecular weights of proteins (Squire, 1964; Kaldor and Gitlin, 1964; Jungwirth and Bodo, 1964).

We used a crude botulin toxin obtained by growing strain No 98 on a Gluzman medium, the strain possessing specific activity of $0.4 \cdot 10^6$-$0.6 \cdot 10^6$ Dlm in one milligram for white mice. Fractionation was performed on a chromatographic column, 600 x x 22 mm in size, filled with G-200 sefadex. The toxin was added to the columns in the volume of one millimeter; the elution was carried out with an isotonic borate buffer (pH 7.3). The fractions were collected on an automatic collector, at 6.5 ml per volume. The outflow rate was 15-18 ml per hour. In the collected fractions the content of nitrous substances was determined according to extinction with the Lowry-Folin reagent, and toxicity (Dlm) on white mice.

For calibration of the column under standard conditions, we filtered a rabbit's serum, crystalline egg albumin, crystalline trypsin and phenol. The serum globulin 19S was eluated on sefadex G-200 columns in the volume of 75-85 ml; serum globulin 7S (molecular weight 160,000) -- in a 115-125 ml volume; serum albumin (molecular weight 65,000) -- 165-175 ml; egg albumin (molecular weight 46,000) -- 190-195 ml; trypsin (molecular weight 24,000) -- 205-215 ml; phenol (molecular weight 94.1) -- in the volume of 240-260 ml.

Bacterial exotoxins, according to the data of Gaurovits (1965), are typical representatives of globular proteins; therefore, the use of proteins with known molecular weight, which also belong to globular proteins, can be justified for calibration of the columns.

In the determination of the molecular weight of the toxin, it is necessary to know only into which of these groups falls the investigated protein following filtration through gel. For a more accurate determination of the molecular weight of proteins, following filtration through sefadex G-200 gel, a calibration curve was drawn (Fig. 1).

Upon fractionation on sefadex G-200 gel, the crude type A botulin toxin was divided into a number of fractions. The elution of proteins with molecular weight of 65,000, and higher, was elicited, according to the results of extinction with Lowry-Folin reagent of 2-3 small protein peaks. The principal mass of nitrous compounds appeared in the elution zone of low-molecular compounds (volume 240-260 milligrams). The obtained results conform with the data concerning the content of total and protein nitrogen in the crude botulin toxin, which has been prepared on the Gluzman medium. Total nitrogen content varied within the range of 700-800 mg%, that of amine nitrogen -- 300 to 380 mg%, protein nitrogen -- 10-15 mg%.

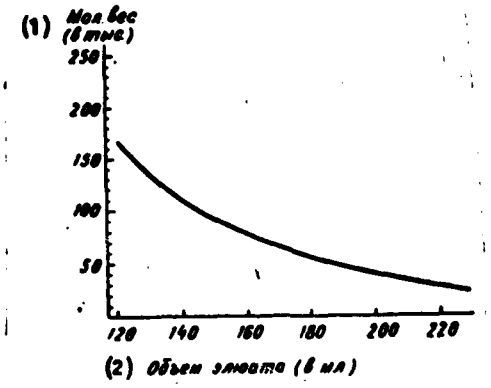

Fig. 1. Curve for Determination of the Molecular Weight of Proteins by Filtration Sefadex G-200 Gel (Column of 600 x 22 mm).

1 -- Mol. weight (in thousands); 2 -- Eluate volume (in milligrams).

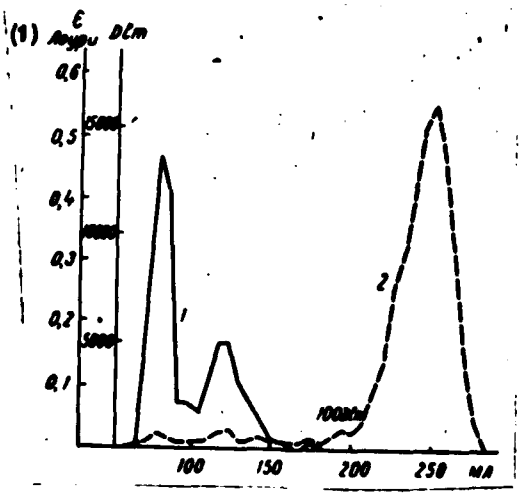

Fig. 2. Separation of Crude Type A Botulin Toxin by Filtration Through Sefadex G-200 Gel (Column 600 x 22).

1 -- Amount of Dlm in one millimeter; 2 -- Extinction with Lowry-Folin reagent.

a -- Lowry.

As seen in Fig. 2 which represents the results of one of the experiments on the separation of botulin toxin, the toxin yield was characterized by two peaks: over 60% of the total toxic activity of the fractions was connected with proteins of large molecular weight, yielding in an easy volume (molecular weight over 200,000); about 30% of toxic activity was connected with proteins of about 160,000 molecular weight. A small activity was also elicited (about 1%), connected with proteins of molecular weight close to 65,000. Analogous picture was observed also in other tests on the separation of botulin toxin type A. The Table cites data of determination in three experiments of the content of protein and of the toxicity and specific activity of various fractions of the toxin. Proteins of fraction 1 possessed the highest specific toxic activity. The activity of fraction 2 and especially of fraction 3 was lower.

Characteristics of Toxin Fractions Obtained Via Fractionation of Type A Botulin Toxin on Sefadex G-200

(1) № опыта	(2) Исходный токсин: (3) количество Dlm в 1 мл	(2) Исходный токсин: (4) содержание азота (в мг%)	(5) Фракция токсина	(6) Объем элюата (в мл)	(7) Токсичность (в Dlm) на 1 мг	(8) Содержание белка по Лоури (в мг%)	(9) Удельная активность (в Dlm на 1 мг азота)
22	$0,4 \cdot 10^6$	720	(10) 1-я	78—90	14 000	1,6	$5,6 \cdot 10^6$
			2-я	110,5—123,5	5 000	2,0	$1,7 \cdot 10^6$
			3-я	169—175,5	100	1,3	$5,0 \cdot 10^4$
23a	$0,4 \cdot 10^6$	760	1-я	78—84,5	10 000	2,0	$3,3 \cdot 10^6$
			2-я	123,5—130	5 000	1,9	$1,7 \cdot 10^6$
			3-я	160,5—167	100	0,6	$1,0 \cdot 10^6$
24a	$0,5 \cdot 10^6$	760	1-я	78—90	15 000	2,6	$3,8 \cdot 10^6$
			2-я	110,5—123,5	7 000	2,6	$1,8 \cdot 10^6$
			3-я	169—182	100	1,0	$6,6 \cdot 10^4$

1 -- No of experiment; 2 -- Initial toxin; 3 -- Amount of Dlm in 1 ml; 4 -- Nitrogen content (in mg%); 5 -- Toxin fraction; 6 -- Volume of the eluate (in ml); 7 -- Toxicity (in Dlm) per milligram; 8 -- Protein content, as per Lowry (in mg%); 9 -- Specific activity (in Dlm per 1 mg of nitrogen); 10 -- 1st...2nd...3rd.

Data on the quantitative ratio of toxicity in the fractions somewhat differ from those obtained by Wagman and Bateman. In their experiments, the fraction with a molecular weight close to 160,000 comprised only about 14%; the rest of the toxin was bound with high molecular proteins. Apparently, these differences were due to the fact that the authors examined a highly purified preparation of botulin toxin, and that in purifying botulin antigens it is the low-molecular toxins which are

eliminated in the first place. It is therefore more expedient to carry out experiments with crude unpurified toxins.

Hence, our investigations confirm the opinion concerning the non-uniform size of the molecules of type A botulin toxin, and establish the quantitative correlations between these molecules in the crude preparations.

Conclusions

1. Toxin molecules in the type A botulin toxin vary as to size.

2. The basic toxic activity in crude preparations is bound with protein of molecular weight over 200,000; one third -- with proteins of molecular weight close to 160,000, and about 1% -- with proteins of molecular weight close to 65,000.

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